Title: The absence of 5-HT_4 receptors modulates depression- and anxiety-like responses and influences the response of fluoxetine in olfactory bulbectomised mice: adaptive changes in hippocampal neuroplasticity markers and 5-HT_{1A} autoreceptor

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Abstract

Preclinical studies support a critical role of 5-HT_4 receptors (5-HT_4Rs) in depression and anxiety, but their influence in depression- and anxiety-like behaviours and the effects of antidepressants remain partly unknown. We evaluated 5-HT_4R knockout (KO) mice in different anxiety and depression paradigms and mRNA expression of some neuroplasticity markers (BDNF, trkB and Arc) and the functionality of 5-HT_{1A}R. Moreover, the implication of 5-HT_4Rs in the behavioural and molecular effects of chronically administered fluoxetine was assessed in naïve and olfactory bulbectomized mice (OBX) of both genotypes. 5-HT_4R KO mice displayed few specific behavioural impairments including reduced central activity in the open-field (anxiety), and decreased sucrose consumption and nesting behaviour (anhedonia). In these mice, we measured increased levels of BDNF and Arc mRNA and reduced levels of trkB mRNA in the hippocampus, and a desensitization of 5-HT_{1A} autoreceptors. Chronic administration of fluoxetine elicited similar behavioural effects in WT and 5-HT_4R KO mice on anxiety-and depression-related tests. Following OBX, locomotor hyperactivity and anxiety were similar in both genotypes. Interestingly, chronic fluoxetine failed to reverse this OBX-induced syndrome in 5-HT_4R KO mice, a response associated with differential effects in hippocampal neuroplasticity biomarkers. Fluoxetine reduced hippocampal Arc and BDNF mRNA expressions in WT but not 5-HT_4R KO mice subjected to OBX. These results demonstrate that the absence of 5-HT_4Rs triggers adaptive changes that could maintain emotional states, and that the behavioural and molecular effects of fluoxetine under pathological depression appear to be critically dependent on 5-HT_4Rs.

Keywords: 5-HT_4 receptors, knockout mice, fluoxetine, anxiety/depression, olfactory bulbectomy.

1. Introduction

Depression is one of the most prevalent major neuropsychiatric diseases, affecting 20% of the population (Hirschfeld, 2012). Dysfunctions in brain serotonin (5-hydroxytryptamine, 5-HT) volume transmission (Descarries et al., 1975) are postulated to be the major basis of depression, but also of almost all mental diseases (Sharp et al., 2007). During the last two decades, studies have mainly investigated the role of the 5-HT_1 and 5-HT_2 receptors but, recently, the 5-HT_4 receptors (5-HT_4Rs) have taken place in this scenario (Conductier et al., 2006; Lucas et al., 2007). Analyses in *postmortem* brain samples from depressed subjects showed a greater density and functionality of 5-HT_4Rs in cortical and striatal areas (Rosel et al., 2004). Moreover, *in vivo* PET imaging studies in humans demonstrated that a reduction in 5-HT_4Rs potential binding in the striatum is associated with a high risk to suffer from major depression (Madsen et al., 2014). Conversely, a moderate reduction in the concentration of 5-HT_4Rs in both the striatum and amygdala was described in patients treated with fluoxetine for three weeks (Haahr et al., 2014). From the preclinical approach, two different animal models of depression, olfactory bulbectomised (OBX) and glucocorticoid heterozygous receptor mice, showed an increase in the expression of 5-HT_4Rs in the ventral hippocampus or striatum, respectively (Licht et al., 2010). In contrast, a down-regulation of 5-HT_4Rs in the ventral and dorsal hippocampus was reported in the Flinders-sensitive line rat model of depression (Licht et al., 2009).

The 5-HT_4Rs are implicated in the mechanism of action of antidepressants (Lucas et al., 2007; Vidal et al., 2014). We have previously reported a down-regulation of 5-HT_4Rs in the striatum and hippocampus of rats chronically treated with fluoxetine (Vidal et al., 2009) and venlafaxine (Vidal et al., 2010). A recent study further described that activation of the 5-HT_4Rs may partly mediate some antidepressant and anxiolytic actions of fluoxetine in predictive behavioural paradigms [tail suspension test (TST) for depression and open-field/elevated plus maze tests for anxiety (Mendez-David et al., 2014)]. In this context, it is noteworthy to mention that some of the neurogenic actions induced by selective serotonin reuptake inhibitors (SSRIs) involve the 5-HT_4Rs (Imoto et al., 2015). Interestingly at a clinical level, a short-term treatment with a 5-HT_4R agonist in rats and long-term administration of SSRIs induced similar antidepressant/anxiolytic actions (Lucas et al., 2007; Pascual-Brazo et al., 2012; Tamburella et al., 2009; Vidal et al., 2014), a behavioural outcome that is associated

with an increased hippocampal proliferation and neural plasticity markers (Pascual-Brazo et al., 2012).

The anatomical localization of 5-HT_4Rs in the brain supports their involvement in depression and anxiety. These receptors are located in different cerebral structures of the limbic system (olfactory tubercles, prefrontal cortex, hippocampus, amygdala, shell of the nucleus accumbens), the basal ganglia including the substantia nigra (Compan et al., 1996; Waeber et al., 1994), where they modulate the release of different neurotransmitters, including acetylcholine, 5-HT, GABA and dopamine (reviewed in Bockaert et al., 2011). Indeed, the 5-HT_4Rs located in the medial prefrontal cortex exert a positive feedback on the firing activity of the dorsal raphe nucleus (DRN) 5-HT neurons (Lucas and Debonnel, 2002; Lucas et al., 2005), the major origin of 5-HT projections and whose activity is admitted to be critical for maintaining a homeostatic brain serotonergic activity. Pharmacological studies have demonstrated that activation of 5-HT_4Rs by selective agonists enhances the electrical activity of the DRN 5-HT neurons and, interestingly, chronic administration of 5-HT_4R agonists does not induce receptor desensitization in the medial prefrontal cortex (Lucas et al., 2005).

Despite these accumulating evidences about the implication of 5-HT_4Rs in depression and in the effects of antidepressants, few studies have investigated the behavioural, neurochemical and/or molecular consequences of the genetic ablation of 5-HT_4Rs. A reduced firing (-50%) of the DRN 5-HT neurons, with changes in both the expression of the 5-HT_{1A}Rs in the DRN and hippocampus and increased levels of the 5-HT transporter (SERT) and mRNA have been reported in 5-HT_4R KO mice (Conductier et al., 2006). Behavioural studies have shown that these mice display abnormal feeding, locomotor and anxiety-like behaviour in response to stress and novelty, seizure susceptibility and long-term memory deficits (Compan et al., 2004; Jean et al., 2007; Jean et al., 2012; Segu et al., 2010). However, whether the 5-HT_4R KO mice display specific anxiety- and depression-like behaviours in different contextual situations (e.g. novelty suppressed feeding paradigm as a conflict-based test, forced swimming test as a behavioural despair situation, and chronic depression/anxiety models) remains to be fully explored. Similarly, little is known about possible adaptive changes in brain neuroplasticity and neurogenesis in the absence of 5-HT_4Rs despite some pharmacological evidences (Imoto et al., 2015; Pascual-Brazo et al., 2012). In this context, brain-derived neurotrophic factor (BDNF)/trkB signalling pathway intervenes

in the physiopathology and treatment of mood disorders, as evidenced by clinical and preclinical studies (Castrén and Rantamäki, 2010; Duman and Monteggia, 2006). Animals display increased levels of BDNF following electroconvulsive shock and treatment with classic antidepressant drugs (Balu et al., 2008; Chen et al., 2001; Nibuya et al., 1995), but also when treated with 5-HT_4R agonists (Pascual-Brazo et al., 2012). The activity-regulated cytoskeleton associated protein (Arc), and other neuroplasticity markers related to dendritic spine density (Peebles et al., 2010), has also been related to depression and antidepressant drug treatments (De Foubert et al., 2004; Li et al., 2015).

Here, we suspected that mice lacking the 5-HT_4Rs could display a depressive- and anxiety-like behaviours, especially in environmental challenges and when subjected to animal models of chronic depression and anxiety. Also we hypothesize that they will show resistance to the behavioural and molecular effects of antidepressants. Therefore, we have performed several behavioural analyses, including fluoxetine treatment in OBX, animal model of chronic depression/anxiety (Linge et al., 2013; Song and Leonard, 2005), in mice lacking 5-HT_4Rs. In addition, the functionality of 5-HT_{1A}R was evaluated using *in vivo* and *in vitro* techniques because the efficacy of chronic antidepressants is 5-HT_{1A}R-dependent (Albert, 2012). Finally, we have extended our analyses by *in situ* hybridization of the BNDF, trkB and Arc mRNA, and hippocampal proliferation.

2. Material and Methods

2.1. Animals and experimental groups

The 5-HT_4R KO and wild-type (WT) mice (3 months old, 25 ± 1 g) from the breeding of 5-HT_4R heterozygote 129SvTer mice (Compan et al., 2004) or 5-HT_4R KO mice crossed were housed (n = 4-5 per cage) in the animal house of the University of Cantabria in a temperature – controlled environment with 12 h light/dark cycle, with food and water available *ad libitum*. All experiments were carried out with the approval of the Animal Care Committee of the Universidad de Cantabria and were performed following the Spanish legislation (Real Decreto 53/2013) and the European Communities Council Directive 2010/63/UE on “Protection of Animals Used in Experimental and Other Scientific Purposes”. Before the initiation of the behavioural

studies, 5-HT_4R stimulated adenylate cyclase assays were performed to ensure the lack of functional 5-HT_4Rs in KO mice (see methods and Fig. S1).

Three different sets of animals were used (Fig. S2). The first set of WT and 5-HT_4R KO mice were subjected to a battery of anxiety and depression-related tests following a time-schedule (Fig. S2); then, they were sacrificed and their brains used for the *in vitro* studies ([^{35}S]GTPγS autoradiography of 5-HT_{1A}R, *in situ* hybridization of BDNF, trkB and Arc, and BrdU immunohistochemistry).

The second set of WT and 5-HT_4R KO mice were chronically administered fluoxetine (160 mg/l in the drinking water, equivalent to 25 mg/kg/day) or vehicle (drinking water) for 14 days and tested in the same battery of anxiety and depression-related tests.

The third set of WT and 5-HT_4R KO mice were subjected to bilateral olfactory bulbectomy (OBX) or sham surgery using procedures previously employed in our studies [(Linge et al., 2013; Linge et al., 2016), supplementary material]. After a 4-weeks recovery period, sham and OBX were tested in the open-field to confirm the development of the typical OBX-induced syndrome. Then, OBX mice of both genotypes were administered fluoxetine (160 mg/l in the drinking water, equivalent to 25 mg/kg/day) or vehicle (drinking water) and tested in the open-field at day 14 and 28 of treatment. Finally, they were sacrificed and their brains used for in situ hybridization of BDNF, trkB and Arc.

2.2. Anxiety and depression tests

Behavioural studies were performed during the light phase, as previously described in detail (Linge et al., 2016). WT and 5-HT_4R KO mice were placed in the experimental room 30 min before the start of each experiment to acclimatize with the exception of the nesting test that was performed during the dark phase with mice placed individually for the session. Behavioural tests were ordered from the least to most stressful one, and leaving an interval between them (usually 2-3 days) to minimize any potential order effects [open-field, light-dark box, sucrose intake, novelty suppressed feeding (NSF) and forced swimming tests (FST)]. Protocols of each test and behavioural testing schedules are described in detail in the supplementary material.

The open-field test was conducted as previously described (Linge et al., 2013; Linge et al., 2016) in order to evaluate the motor reactivity to novelty and anxiety-related parameters (time and distance travelled in the central area).

The light-dark box test was performed as previously described (Clément et al., 2009). Each mouse was initially placed on the dark side of the box and the time and number of entries into each zone were recorded and analysed during 5 min.

The sucrose intake test that represents an "hedonic" index, was performed as previously described (Linge et al., 2016). Mice were deprived of any drink solution for 24 h. The next day, we quantified the amount of consumed sucrose solution (1%) by each animal during 1 h.

The nesting test was performed as previously reported (Deacon, 2006), which evaluates an apathetic and self-neglect behaviour (Pedersen et al., 2014). At the beginning of the dark phase, mice were individually housed and a 3 g piece of cotton was placed inside the cage. The next day, a blind and trained observer scored the nest production according to a 1 to 5 points scale.

The NSF was performed as previously described (Linge et al., 2013). The latency (in seconds) to eat a pellet placed in the centre of the open-field was evaluated following 24 h food deprivation. Food consumption was also evaluated in mice´s home-cages (immediately after the NSF test).

The FST permits us to evaluate behavioural despair, as previously described (Porsolt et al., 1977). A blind and trained observer manually scored three behavioural parameters (immobility, swimming, climbing) on video-recorded sessions.

2.3. 8-OH-DPAT-induced hypothermia in mice

The protocol was adapted from Zazpe et al., (2006). The experiments were carried out in a room equipped with a thermostat (21.0 ± 0.5ºC) between 10:00 am and 14:00 pm. The body temperature was evaluated for a period of 15 s, or until a stable reading was obtained, by inserting a thermoelectric probe into the rectum (room temperature of 20.0 ± 0.1°C). Initially, three measurements were made at 20 min intervals considering the average of the last two determinations as basal temperature value. Then, 8-OH-DPAT (1

mg/kg) was injected intraperitoneally and the body temperature was evaluated at 20 min.

2.4. In situ hybridization

The brains of mice were rapidly removed and frozen immediately on dry ice and then stored at -80°C until sectioning. Coronal brain 14 µm thick sections from WT and 5-HT_4R KO mice were cut at -20°C using a microtome cryostat and thaw-mounted in slices and stored at -20°C (for [^{35}S]GTPγS binding assay) or -80°C (for *in situ* hybridization).

The protocol was adapted from Castro (Castro et al., 2003a), using oligonucleotides complementary to BDNF mRNAs 5'-GGTCTCGTAGAAATATTGGTTCAGTTGGCCTTTTGATACCGGGAC-3' (Vaidya et al., 2001) and trkB mRNAs 5´-CCTTTCATGCCAAACTTGGAATGTCTCGCCAACTTG- 3' (Madhav et al., 2001) and Arc 5'-GCAGCTTCAGGAGAAGAGAGGATGGTGCTGGTGCTGG-3' (Kelly et al., 2008) were 3´end-labelled with [^{35}S]dATP using terminal deoxynucleotide transferase. Finally, 250000 c.p.m./slide were mixed with hybridization buffer and incubated with brain sections (supplementary materials). The specific distribution of mRNA encoding trkB receptors and BDNF and Arc in the whole brain was consistent with previous studies (Kelly et al., 2008; Madhav et al., 2001; Vaidya et al., 2001).

2.5. [^{35}S]GTPγS autoradiography of 5-HT_{1A}R

Labelling of brain sections (obtained as described above, see 2.4.) with [^{35}S]GTPγS was carried out as previously described (Castro et al., 2003b) in order to evaluate the functionality of 5-HT_{1A}R, using the selective agonist 8-OH-DPAT (10 µM). The non-specific binding was determined in the presence of 10 µM guanosine-5-O-(3-thio)triphosphate (GTPγS, supplementary material).

Labelling of coronal brain sections visualized on autoradiograms were analysed and quantified ([^{35}S]GTPγS binding) or semi-quantified (*in situ* hybridization) using a computerized image analysis Scion Image software (Scion Corporation, MD, USA). Optical density values were calibrated using ^{14}C microscales, and expressed in nCi/g of estimated tissue equivalent.

2.6. BrdU-immunohystochemistry

BrdU staining was performed as previously described (Mostany et al., 2008). Free floating coronal sections were incubated 2 h in 50% formamide/2x SSC (saline sodium citrate) buffer at 65°C, 30 min in 2N HCl, and 10 min in 0.1M borate buffer. After PBS washing, sections were incubated in 1% H_2O_2 for 30 min, blocked 30 min in PBS/0.2% Triton X-100/5% goat serum and incubated with monoclonal mouse anti-BrdU overnight at 4°C. After PBS-TS washes, sections were incubated 2 h with biotinylated goat anti-mouse Fab Fragment IgG secondary antibody, followed by amplification with avidin-biotin complex (Vector Laboratories). $BrdU^+$ cells were counted using a light microscope (Carl Zeiss Axioskop 2 Plus) (see supplementary material).

2.7. Drugs and chemicals

[^{35}S]dATP(2' Deoxyadenosine 5'-(α- thio) Triphosphate, [^{35}S] Guanosine 5'-(γ- thio) Triphosphate (GTPγS), at a specific activity of 1250 Ci/mmol was purchased from Perkin Elmer. Zacopride hydrochloride and fluoxetine hydrochloride were purchased from Tocris Bioscience, and 8-OH-DPAT from Sigma Aldrich. All other chemicals used were of analytical grade.

2.8. Data analysis and statistics

The statistical analyses were performed using Student's *t*-test, Mann-Whitney U test or two-way ANOVA. When effects of independent variables (treatment, genotype), or interactions were significant, one-way ANOVAs (treatment, genotype) were performed followed by *post-hoc* test when appropriated. The type of statistical analysis is indicated in the results section and in the legends of figures. The level of significance was set at $p < 0.05$ (Table S1). Graphs editing and statistical analyses were performed using the GraphPad Prism Software (GraphPad, San Diego, CA, USA).

3. Results

3.1. 5-HT$_4$R KO mice display anhedonia and a context-dependent anxiety-like response

In the open-field test, 5-HT$_4$R KO mice presented lower central activity as evidenced by a reduction in the central time (46.7 ± 3.0 s) compared with WT counterparts (60.4 ± 5.5 s, $p < 0.05$, Fig. 1A), with a similar number of entries in the central area (WT: 28.4 ±

1.5 *vs* KO: 24.0 ± 1.9, Fig. 1B). It was not associated with altered locomotion because mice of both genotypes travelled a similar total distance (WT: 20.7 ± 1.5 m *vs* KO: 21.4 ± 0.9 m, Fig. 1C). No difference between the mice of both genotypes was also observed in the LDB (Fig. 1D). Two-weeks treatment with fluoxetine induced a significant reduction of the central time in mice of both genotypes (WT-flx: 35.1 ± 4.6 s *vs* WT, $p < 0.01$; KO-flx: 24.9 ± 6.7 s *vs* KO, $p < 0.01$, Fig. 1A). Accompanied with a significant reduction of the central entries (WT-flx: 17.0 ± 2.0 *vs* WT, $p < 0.01$; KO-flx: 12.9 ± 2.6 *vs* KO, $p < 0.01$, Fig. 1B) but no change was observed in the LDB (Fig. 1D).

Additionally, 5-HT$_4$R KO mice did not show significant changes in the latency to feed following the NSF test (WT: 203.9 ± 35.6 s *vs* KO: 219.7 ± 29.8 s, Fig. 1E). Chronic treatment with fluoxetine induced a similar reduction of the latency to feed in mice of both genotypes (WT-flx: 102.4 ± 11.4 *vs* WT, $p < 0.05$; KO-flx: 118.3 ± 17.9 s *vs* KO, $p < 0.05$, Fig. 1E). Mice of both genotypes consumed a similar amount of food when returned to the home-cage after the NSF test in the basal conditions and following chronic fluoxetine treatment (Fig. 1F).

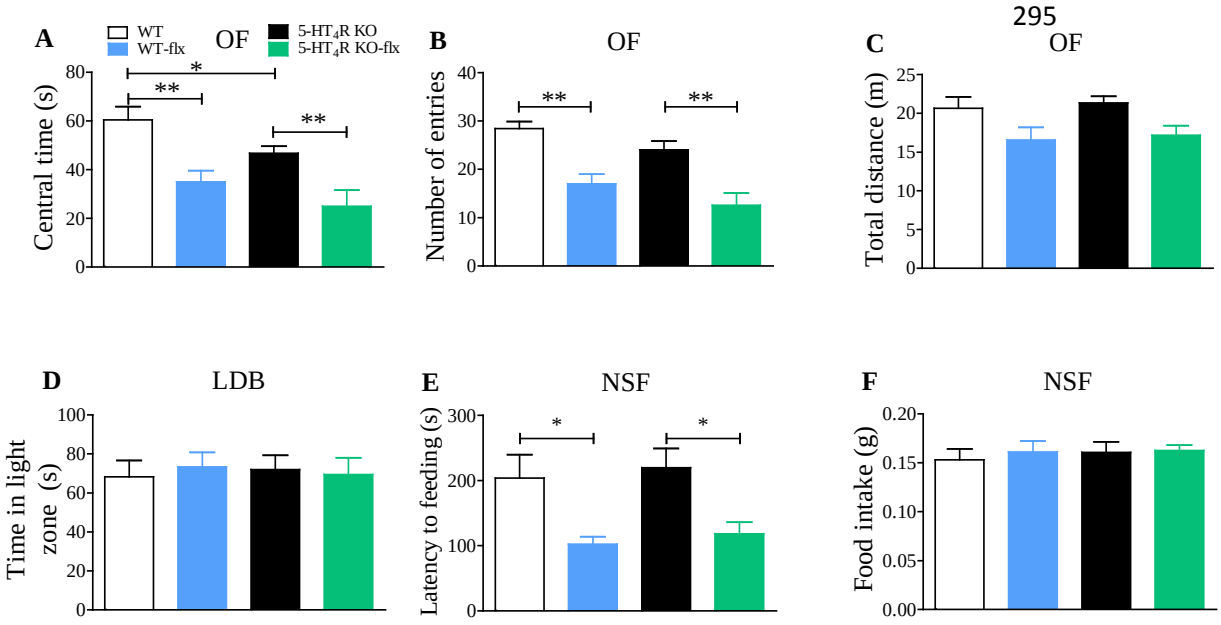
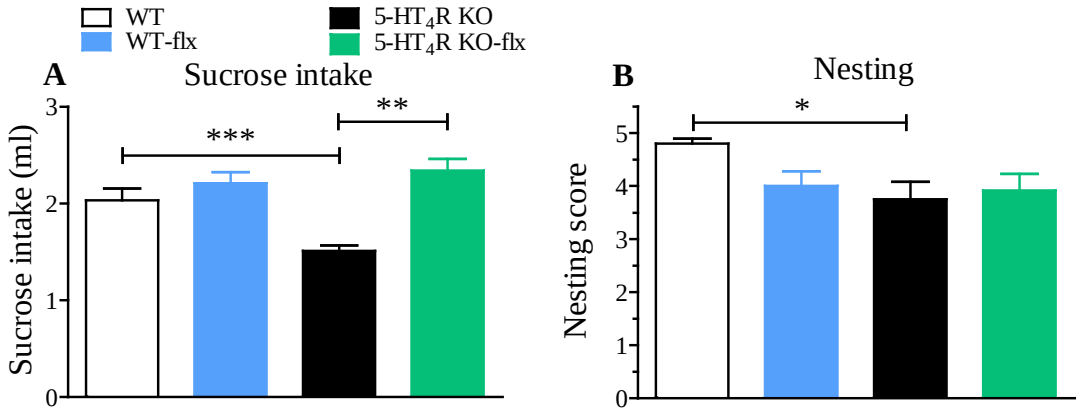


Figure 1. ***Behaviour of WT and 5-HT$_4$R KO mice in different anxiety-related paradigms.*** In the open-field test (5 min), 5-HT$_4$R KO mice spent less time than WT counterparts in the central zone, and chronic fluoxetine induced a significant reduction of the central time spent in mice of both genotypes **(A).** WT and 5-HT$_4$R KO exhibited a similar number of central entries, and chronic fluoxetine induced a similar effect in mice of both genotypes **(B).** Total distance was not significantly different between mice of both genotypes and following fluoxetine treatment **(C)**. No significant changes were

found in the light-dark box test. **(D)** The latency to feed between WT and 5-HT$_4$R KO mice was not different, and a similar reduction was found in mice of both genotypes following the chronic fluoxetine treatment **(E)**. Post-NSF test food intake was not different between mice of both genotypes and following fluoxetine treatment **(F)**. Data are mean ± SEM of *n* = 13-18 mice per group. Two-way ANOVA revealed a main effect of the genotype and treatment on the time spent in the central part of the open-field ($F_{(1,58)} = 6.0$, $p < 0.05$ for genotype effect and $F_{(1,58)} = 23.2$, $p < 0.001$ for treatment effect) **(A)**. Also, a main effect of the genotype ($F_{(1,58)} = 5.1$, $p < 0.05$) and treatment ($F_{(1,58)} = 34.2$, $p < 0.001$) was found on the number of central entries in the open field **(C)** and a treatment effect was found in the latency to feed in the novelty suppressed feeding ($F_{(1,50)} = 14.4$, $p < 0.001$) **(E)**. *$p < 0.05$ and **$p < 0.01$ (Newman-Keuls post hoc test).

5-HT$_4$R KO animals showed a lower sucrose intake than WT counterparts (KO: 1.5 ± 0.1 ml *vs* WT: 2.0 ± 0.1 ml, $p < 0.001$, Fig. 2A), an outcome that was reversed by chronic fluoxetine (KO-flx: 2.3 ± 0.1 ml *vs* KO, $p < 0.01$, Fig. 2A). Additionally, an impaired nesting performance was observed in 5-HT$_4$ R KO mice (nesting score of KO: 4.1 ± 0.3 *vs* WT: 4.8 ± 0.1, $p < 0.05$, Fig. 2B). In the FST, mice of both genotypes exhibited similar immobility (WT: 206.4 ± 6.2 s *vs* KO: 204.7 ± 9.1 s, Fig. 2C), swimming (WT: 30.3 ± 5.4 s *vs* KO: 33.1 ± 8.5 s, Fig. 2D) and climbing (WT: 3.4 ± 1.1 s *vs* KO: 2.1 ± 1.1 s, Fig. 2E) scores. Chronic fluoxetine treatment induced similar reductions in immobility (WT-flx: 161.0 ± 12.0 s *vs* WT, $p < 0.01$; KO-flx: 153.3 ± 12.6 s *vs* KO, $p < 0.01$, Fig. 2C), and increases in both swimming (WT-flx: 63.9 ± 9.8 s *vs* WT, $p < 0.05$; KO-flx: 76.1 ± 11.1 s *vs* KO, $p < 0.01$, Fig. 2D) and climbing (WT-flx: 15.1 ± 3.2 s *vs* WT, $p < 0.001$; KO-flx: 11.6 ± 2.4 s *vs* KO, $p < 0.05$, Fig. 2E) behaviours in mice of both genotypes.



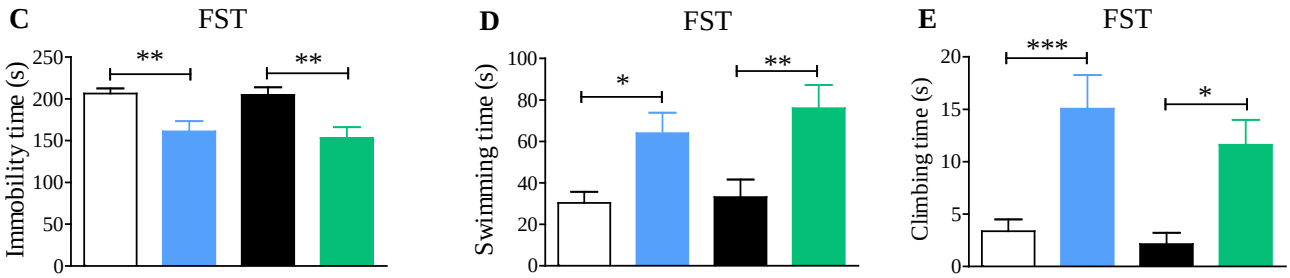


Figure 2. ***Behaviour of WT and 5-HT$_4$R KO mice in different depression-related paradigms.*** 5-HT$_4$R KO mice exhibited reduced sucrose intake that was reversed by chronic fluoxetine treatment (**A**), and reduced nesting behaviour (**B**) compared with WT mice. No differences were observed between mice of both genotypes in all FST parameters [immobility (**C**), swimming (**D**) and climbing (**E**)]. In the sucrose intake test, two-way ANOVA analyses revealed a main effect of treatment ($F_{(1,60)} = 22.7$, $p < 0.001$), and a main effect of genotype x treatment interaction ($F_{(1,60)} = 9.5$, $p < 0.01$). In the nesting test, two-way ANOVA analysis revealed a main effect of genotype ($F_{(1,63)} = 4.4$, $p < 0.05$). In the FST, chronic fluoxetine treatment induced similar effects in all the measured outcomes in mice of both genotypes; two-way ANOVA analyses revealed a main effect of treatment (immobility: $F_{(1,51)} = 21.6$, $p < 0.001$; swimming: $F_{(1,51)} = 18.3$, $p < 0.001$; climbing: $F_{(1,50)} = 20.7$, $p < 0.001$). Data are mean ± SEM of n = 13-20 mice per group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (Newman-Keuls post hoc test).

3.2. 8-OH-DPAT-induced hypothermia following chronic fluoxetine treatment

The functionality of 5-HT$_{1A}$Rs was assessed *in vivo* by measuring 8-OH-DPAT-induced hypothermia (Fig. 3). A similar decrease of rectal temperature in vehicle-treated mice of both genotypes was observed at 20 min following the administration of 8-OH-DPAT (WT: -2.9 ± 0.3ºC *vs* KO: -2.7 ± 0.3ºC). As expected, chronic treatment with fluoxetine induced a reduction of the hypothermia induced by 8-OH-DPAT administration in mice of both genotypes (WT- flx: -1.8 ± 0.1ºC *vs* WT, $p < 0.01$; KO-flx: -1.1 ± 0.2ºC *vs* KO, $p < 0.001$). This reduction was lower in fluoxetine-treated 5-HT$_4$R KO compared with fluoxetine-treated WT mice ($p < 0.05$).

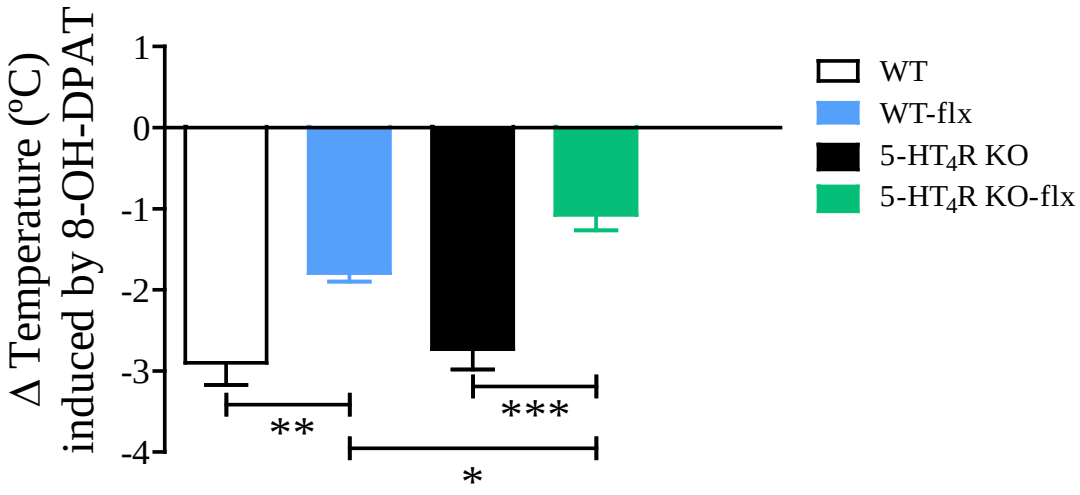


Figure 3. *Effect of chronic administration of fluoxetine on 8-OH-DPAT-induced hypothermia paradigm*. Chronic administration of fluoxetine induced a reduction of the hypothermic effect of 8-OH-DPAT in mice of both genotypes. Note that 5-HT$_4$R KO mice treated chronically with fluoxetine exhibited a significant lower 8-OH-DPAT-induced hypothermic effect respect to WT counterparts. Two-way ANOVA analysis revealed a main effect of the genotype ($F_{(1,19)} = 4.7$, $p < 0.05$), treatment ($F_{(1,19)} = 42.3$, $p < 0.001$) but not a main effect of the genotype x treatment interaction. Data are mean ± SEM of n = 5-7 mice per group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Newman-Keuls post hoc test).

3.3. Reduced G-protein signalling of presynaptic 5-HT$_{1A}$R in 5-HT$_4$R KO mice

The 5-HT$_{1A}$R activity was also assessed *in vitro* by measuring 8-OH-DPAT stimulated [^{35}S]GTPγS binding in brain sections from mice of both genotypes. As shown in Table 1 and Fig. 4, 8-OH-DPAT-induced stimulation of specific [^{35}S]GTPγS binding was lower in the DRN of 5-HT$_4$R KO mice compared with WT counterparts (-28.3%, $p < 0.05$). An increase in basal [^{35}S]GTPγS binding values (nCi/g tissue) was also detected in the brain of 5-HT$_4$R KO mice at the level of both the DRN (WT: 301.8 ± 6.8 *vs* KO: 360.5 ± 19.3, $p < 0.05$) and the prefrontal cortex (WT: 285.9 ± 25.5 *vs* KO: 377.8 ± 22.4, $p < 0.05$). No significant differences were found either in basal and stimulated [^{35}S]GTPγS binding in the others areas analysed (the hippocampus and entorhinal cortex).

Table 1. Absolute values (nCi/g tissue) of specific [^{35}S]GTPγS binding induced by 8-OH-DPAT. DRN: dorsal raphe nucleus, PFrCx: prefrontal cortex, CA1: CA1 field of the hippocampus, CA3: CA3 field of the hippocampus, DG: dentate gyrus of the hippocampus and EntCx: entorhinal cortex. Data are mean ± SEM, number of animals per condition in brackets (*n*). **p* < 0.05 (Student´s *t* - test, unpaired data).

Specific [^{35}S]GTPγS binding induced by 8-OH-DPAT (nCi/g tissue)		
Brain areas	WT	5-HT$_4$R KO
DRN	181.9 ± 9.7 (7)	130.4 ± 18.3 (7)*
PFrCx	82.4 ± 17.0 (7)	94.8 ± 19.0 (6)
CA1	194.7 ± 25.8 (7)	222.8 ± 28.7 (7)
CA3	68.7 ± 17.5 (5)	69.4 ± 19.6 (6)
DG	58.8 ± 22.7 (5)	64.4 ± 25.9 (7)
EntCx	167.5 ± 13.5 (6)	218.5 ± 18.8 (7)

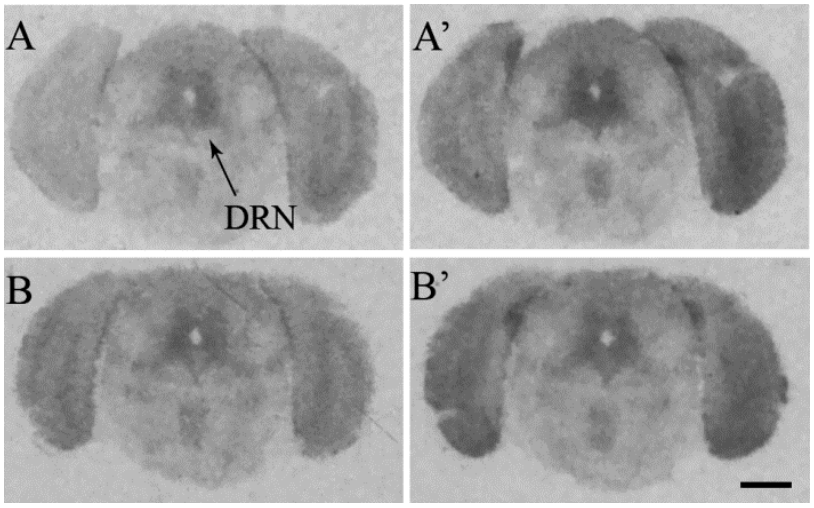


Figure 4. Autoradiographs in transverse midbrain sections of 8-OH-DPAT stimulated [^{35}S]GTPγS binding. Upper: WT mice, basal (A) and stimulated (A') binding. Lower: 5-HT$_4$R KO mice, basal (B) and stimulated binding (B'). DRN: dorsal raphe nucleus. Scale bar = 1 mm.

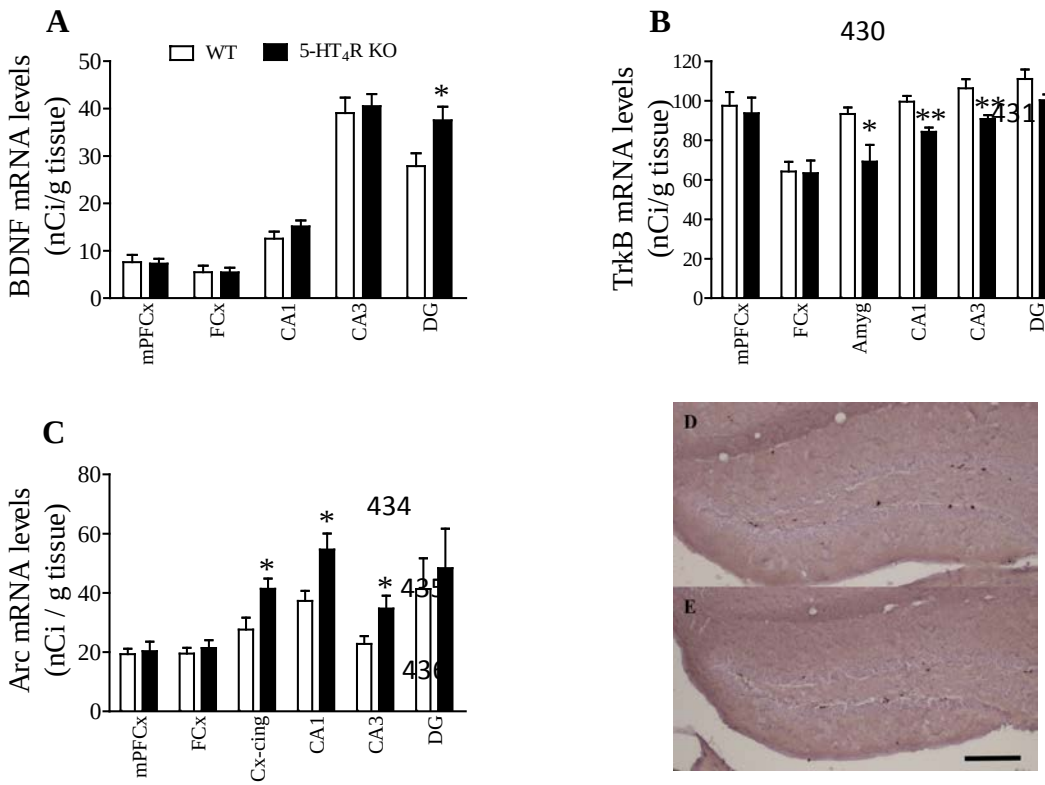
3.4. Altered BDNF, trkB and Arc expression levels in 5-HT$_4$R KO mice

Differences between WT and 5-HT$_4$R KO mice were detected in the levels of both BDNF and trkB mRNA. The highest levels of BDNF and trkB mRNA were observed in

the hippocampus of both WT and 5-HT$_4$R KO mice. The 5-HT$_4$R KO mice showed higher increases in the levels of BDNF mRNA in the dentate gyrus (DG) of the hippocampus than WT mice (~35%; $p < 0.05$, Fig. 5A), which was not associated with significant changes in the levels of trkB mRNA (Figs. 5B and F). Additionally, 5-HT$_4$R KO mice exhibited reduced levels of trkB mRNA in the other hippocampal fields (CA1 and CA3: ~ 15 %, $p < 0.01$), and in the amygdala (~ 26 %, $p < 0.05$) compared with WT mice (Figs. 5B and F). No differences were detected in the levels of trkB and BDNF mRNA in the examined areas of the cerebral cortex between mice of both genotypes (Figs. 5A, B and F). Finally, the levels of mRNA encoding Arc (Figs. 5C and F) were increased in the CA1 and CA3 hippocampal fields and the cingulate cortex in 5-HT$_4$R KO mice (~ 50%) compared with WT mice ($p < 0.05$).

3.5. Absence of impaired hippocampal proliferation in 5-HT$_4$R KO mice

Hippocampal proliferation was evaluated as the incorporation of the thymidine analogue BrdU in the subgranular zone of the DG. A similar number of BrdU immunolabelled cells was detected in both 5-HT$_4$R KO (1522.0 ± 149.3 BrdU$^+$ cells, Fig. 5E) and WT (1483.0 ± 109.3 BrdU$^+$ cells, Fig. 5D) mice.



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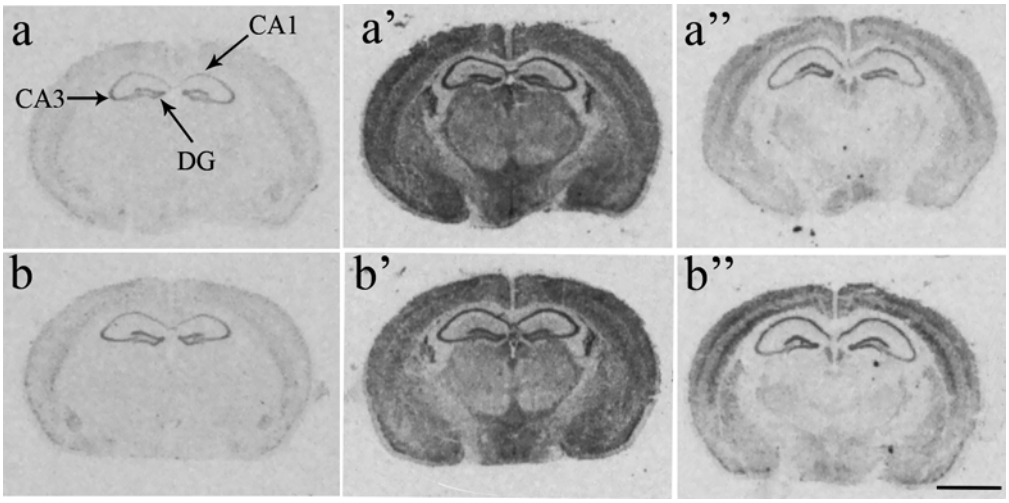

Figure 5. Changes in neuroplasticity markers in 5-HT$_4$R KO mice. Levels of BDNF (**A**), trkB (**B**) and Arc (**C**) mRNA. Data are mean ± SEM, *n* = 6-7 mice per group. mPFCx: medial prefrontal cortex, FCx: frontal cortex, Amyg: amygdala, CingCx: cingulate cortex, CA1 and CA3: CA1 and CA3 fields of the hippocampus and DG: dentate gyrus. **p* < 0.05 and ***p* < 0.01 *vs* WT, Student´s *t*-test, unpaired data. Illustrations showing BrdU immunopositive cells in the DG in WT **(D)** and 5-HT$_4$R KO (**E**) mice, scale bar: 20 µm. (**F**) Distribution of BDNF (**a, b**), trkB (**a', b'**) and Arc (**a'', b''**) mRNA visualized on autoradiographs in transverse brain sections from WT (upper) and 5-HT$_4$R KO mice (lower) at the level of the dorsal hippocampus, following *in situ* hybridization. Scale bar: 2 mm.

3.6. Chronic fluoxetine failed to reverse OBX-induced syndrome in 5-HT$_4$R KO mice

Following four weeks of OBX surgery, mice of both genotypes displayed similar locomotor hyperactivity, as evidenced by the increased total distance travelled in the open-field (WT-sham: 18.3 ± 0.9 m *vs* WT-OBX: 25.4 ± 2.8 m, $p < 0.05$; KO-sham: 16.7 ± 1.0 m *vs* KO-OBX: 24.4 ± 3.2 m, $p < 0.05$, Fig. 6A). A similar temporal pattern of locomotor activity was observed in mice of both genotypes before and after sham or OBX surgery (Figs. S3A and B). This hyperactivity was related to an enhanced thigmotaxis as reflected by an increased ambulation at the periphery of the open-field (WT-sham: 13.8 ± 1.2 m *vs* WT-OBX: 23.6 ± 2.7 m, $p < 0.05$; KO-sham: 12.4 ± 1.2 m *vs* KO-OBX: 22.4 ± 3.1 m, $p < 0.01$) (Fig. S4A).

Mice of both genotypes exhibited similar anxiety-like behaviour induced by OBX, as evidenced by a reduced activity in the central part of the open-field (central time: WT-sham: 49.8 ± 10.3 s *vs* WT-OBX: 9.2 ± 1.4 s, $p < 0.001$; KO-sham: 42.5 ± 6.1 s *vs* KO-

OBX: 13.0 ± 3.0 s, $p < 0.001$, Fig. 6B). Similar readouts were observed in other central parameters (Figs. S4B and C).

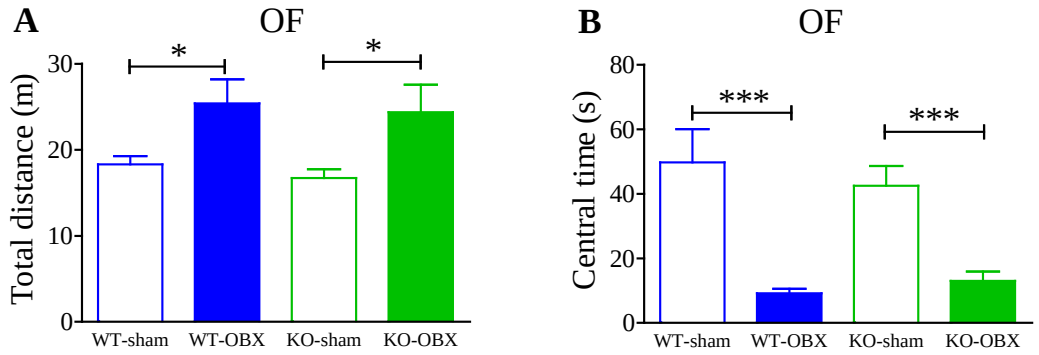

Figure 6. Similar responses to olfactory bulbectomy in WT and 5-HT$_4$R KO mice. Total distance (**A**) and central time (**B**) in the open-field following 4 weeks post surgery. Data represent mean ± SEM of n = 7-8 mice per group. Two-way ANOVA revealed a main effect of the surgery on the total distance travelled ($F_{(1,26)} = 9.4$, $p < 0.01$) and on the time spent in the central part of the open-field ($F_{(1,26)} = 35.1$, $p < 0.001$) but no significant surgery x genotype interaction. *$p < 0.05$ and ***$p < 0.001$ (Newman-Keuls post hoc test).

Considering similarities in OBX-syndrome between both WT and 5-HT$_4$R KO mice, fluoxetine was chronically administrated for 28 days. Animals were again tested in the open-field at days 14 and 28 (Fig. 7). A *post hoc* analysis showed a total reversal of the OBX-induced hyperactivity in fluoxetine-treated WT-OBX mice. Indeed, chronic fluoxetine treatment reduced, in a time-dependent manner, the characteristic OBX-induced locomotor hyperactivity to values similar to those observed in the respective sham-operated mice (WT-OBX-fluoxetine: 10.8 ± 2.4 m *vs* WT-OBX: 23.6 ± 2.7 m, $p < 0.05$) following 28 days of treatment. In contrast, chronic administration of fluoxetine failed to reverse OBX-induced hyperactivity in 5-HT$_4$R KO mice [achieving only 15% of reduction in the total distance travelled, (Fig. 7A)]. Additionally, chronic administration of fluoxetine failed in eliciting a positive effect in the habituation to novelty in 5-HT$_4$R KO mice (Fig. 7B).

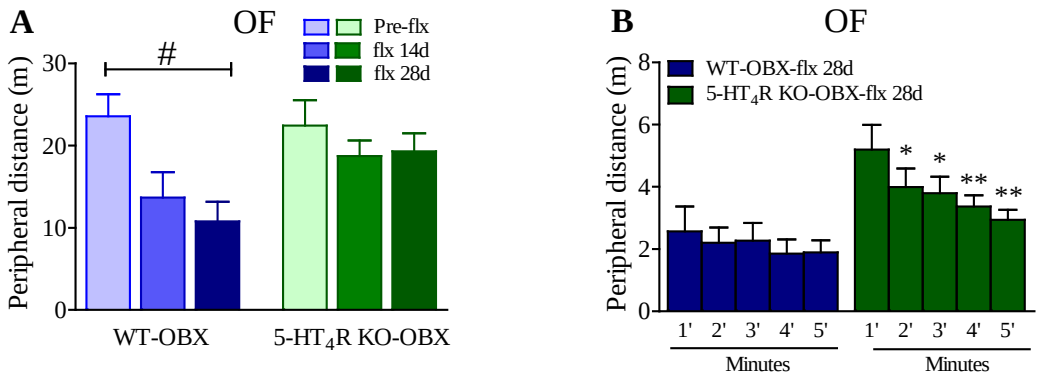

Figure 7. Chronic fluoxetine failed to reverse OBX syndrome in 5-HT$_4$R KO mice. Total peripheral distance (OF, 5 min session) evaluated before and following fluoxetine (flx) treatment; Two-way ANOVA revealed a genotype x time interaction on the distance travelled at the periphery [$F_{(1,26)} = 7.1$, $p < 0.01$ (**A**)]. Peripheral distance per one min intervals at day 28 of fluoxetine treatment (flx 28d); Two-way ANOVA revealed a significant effect of time ($F_{(4,52)} = 4.7$, $p < 0.01$) and genotype [$F_{(1,13)} = 6.6$, $p < 0.05$ **(B)**]. Data are mean ± SEM of n = 7-8 mice per group. $^{\#}p < 0.05$ *vs* pre-flx; $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs* 1 min intervals (Newman-Keuls post hoc test). Pre-flx: before the treatment with fluoxetine; flx 14d and flx 28d: 14 and 28 days of fluoxetine treatment.

3.7. Differential changes in BDNF and Arc mRNA in chronic fluoxetine-treated 5-HT$_4$R KO-OBX mice

In order to set out to explore the neural substrates related to the behavioural outcome of 5-HT$_4$R KO-OBX mice chronically treated with fluoxetine, we assayed the levels of BNDF and Arc mRNA. A differential regulation in plasticity makers was observed between WT and 5-HT$_4$R KO mice. In WT-OBX mice, the chronic fluoxetine treatment induced decreases in the levels of BNDF mRNA in the DG (21%, $p < 0.05$ *vs* WT-OBX, Fig. 8A) and CA3 (31%, $p < 0.05$ *vs* WT-OBX, Fig. 8B) hippocampal areas examined, but not in 5-HT$_4$R KO-OBX mice. The antidepressant exerted a similar effect in the levels of BNDF mRNA in CA1 hippocampal field in mice of both genotypes subjected to OBX (Fig. 8C).

In addition, chronic fluoxetine treatment induced decreases in the levels of Arc mRNA in both WT and 5-HT$_4$R KO-OBX mice in the DG (Fig. 8D) and CA3 (Fig. 8E) hippocampal areas. However, the antidepressant did reduce the levels of Arc mRNA in the CA1 in WT-OBX (56%, $p < 0.01$ WT-OBX-FLX *vs* WT-OBX), but not in 5-HT$_4$R KO mice (Fig. 8F).

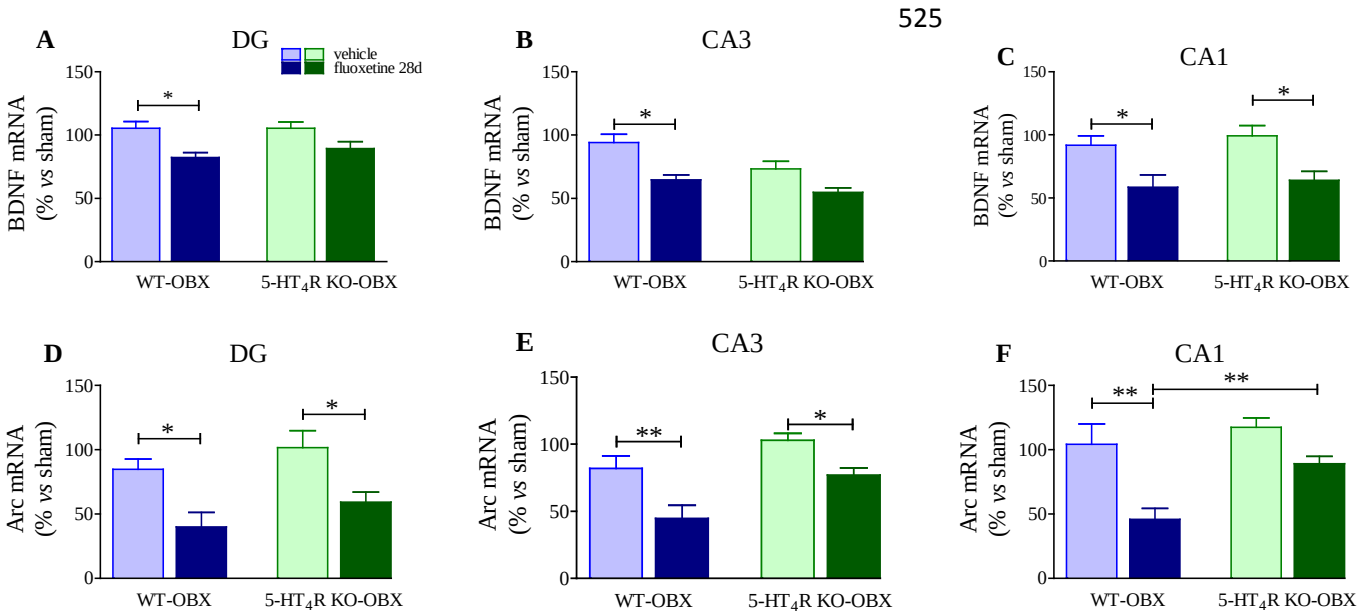

Figure 8. mRNA expression of neuroplasticity markers in chronic fluoxetine-treated OBX mice. Levels of BDNF (**A, B, C**) and Arc (**D, E, F**) mRNA. Two-way ANOVA analyses revealed a significant main effect of treatment on the levels of BDNF mRNA in the DG ($F_{(1,30)} = 16.1$, $p < 0.001$) and in the CA3 ($F_{(1,31)} = 14.0$, $p < 0.001$) hippocampal areas. Two-way ANOVA analyses also revealed a significant main effect of treatment ($F_{(1,31)} = 13.9$, $p < 0.001$) and genotype ($F_{(1,31)} = 5.9$, $p < 0.05$) on the levels of Arc mRNA in the CA1 hippocampal field. Data are mean ± SEM of $n = 7$-8 mice per group. *$p < 0.05$ and **$p < 0.01$ (Newman-Keuls post hoc test).

4. Discussion

The present study shows that 5-HT$_4$R KO mice display anhedonia and a context-dependent anxiety-like behaviour, with responses to the OBX syndrome similar as those detected in WT mice. A critical present finding is the lack of response of 5-HT$_4$R KO mice to the behavioural and molecular antidepressant effects of fluoxetine in the animal model of chronic depression/anxiety (*e.g.* OBX).

Among all tests used to evaluate the potential depressive-like state of the 5-HT$_4$R KO mice, we detected that these mutant animals consumed less sucrose. It suggests an

anhedonic-like behaviour and a specific involvement of 5-HT$_4$Rs in one of the behavioural traits of depression-like behaviour, an outcome reversed by 2-weeks treatment with fluoxetine. Accordingly, the mutant mice exhibited a reduction in the nesting score, another behavioural outcome that might reflect both apathetic and anhedonic-like behaviour. However, results in the forced swimming test indicate that 5-HT$_4$R KO mice are not more prone to show higher behavioural despair or learned helplessness than their WT counterparts. These findings appears to be in disagreement with the pharmacological studies reporting a reduced forced swimming test immobility following acute administration of partial 5-HT$_4$R agonists in rats (Lucas et al., 2007). This could be due to (*i*) compensatory neuroplasticity processes that may install gradually over development in the 5-HT$_4$R constitutive KO mice [*e.g.* adaptive changes in serotoninergic system (Conductier et al., 2006), present study], (*ii*) methodological differences (animal species and different FST protocols) and/or (*iii*) because RS67333 is also a partial agonist that could induced different effects depending on the dose used. All this could contribute to the similar response in the FST and also explain the same effect of fluoxetine observed in mice of both genotypes in this experimental paradigm (Cryan et al., 2005). In addition, the differential behaviour of 5-HT$_4$R KO mice in the FST *vs* sucrose/nesting paradigms could be explained by the participation of different brain areas involved in each particular paradigm. In fact, high concentration of 5-HT$_4$Rs has been detected in the shell of the nucleus accumbens in rats and mice (Compan et al., 1996; Jean et al., 2007). There, they intervene in motivation for foods and influence reward processes (Jean et al., 2007; Jean et al., 2012) through the activation of the cAMP/PKA/pCREB pathway (reviewed in Compan et al., 2015). CREB overexpression in the nucleus accumbens reduces the rewarding effects of sucrose (Barrot et al., 2002). And, the ability of cocaine to induce CREB phosphorylation is absent in the nucleus accumbens of the 5-HT$_4$R KO mice (reviewed in Compan et al., 2015), reinforcing the fact that the absence of 5-HT$_4$R favours an anhedonic behaviour (present study). Also, rats subjected to maternal deprivation exhibit a strong correlation between 5-HT$_4$Rs mRNA in the hippocampus and anhedonia-like behaviour (Bai et al., 2014). The absence of 5-HT$_4$Rs in the nucleus accumbens and the hippocampus may likely account for anhedonia-like behaviour of 5-HT$_4$R KO mice. Among the different neuroplasticity markers that have been analysed in the present study, results revealed increased levels of Arc mRNA in the hippocampus and the cingulate cortex of 5-HT$_4$R KO mice. This might support their anhedonia since enhanced expression of Arc mRNA in cortical and

hippocampal areas has been described in rodents subjected to social defeat (Coppens et al., 2011) and chronic unpredictable mild stress (Boulle et al., 2014). There is also a reduced concentration in the 5-HT_{1A}R in the dorsal hippocampus of 5-HT_4R KO mice (Conductier et al., 2006). The participation of these hippocampal 5-HT_{1A}Rs in anhedonia and, especially, in the antidepressant effects of fluoxetine must also be considered. Indeed, they may participate in the anti-anhedonic effect of chronic treatment with fluoxetine observed in 5-HT_4R KO mice.

Depression- and anxiety-like behaviours rarely exist independently, and here, in the open-field test, 5-HT_4R KO mice presented a reduced central time, suggesting an increased anxiety in good accordance with a previous report (Compan et al., 2004). However, in other tests, which also permit us to evaluate anxiety-like responses under different environmental challenges (light-dark box and novelty suppressed feeding), 5-HT_4R KO mice exhibited an anxiogenic response similar to that observed in WT mice. It is well known that different aspects of emotionality are covered by the umbrella term "anxiety" (File, 1992). This discrepancy between the findings in the open-field versus the light-dark box/novelty suppressed feeding tests could be explained when considering the participation of distinct/complementary brain areas that may be differentially engaged in each particular test and/or the particular profile of fluoxetine´s effects in anxiety-related paradigm depending on the dose administered (Dulawa et al., 2004). The behavioural findings following chronic fluoxetine treatment accredit this hypothesis since its chronic administration produced opposite effects in the open-field and the novelty suppressed feeding. In fact, fluoxetine induced an anxiogenic effect in the former but a marked anxiolytic effect in the latter test. Moreover, the light-dark box rather than open-field is a more appropriate approach to assess permanent anxiety [“trait anxiety”, (File, 1992; Ramos, 2008)], and the novelty suppressed feeding test more reliable evaluation of the mice´s performance under a conflictive-aversive context (Belzung and Griebel, 2001). All the above findings suggest that the *Htr*4 gene deficit could enhance anxiety state in a context-dependent manner, but not an anxiety trait, as seen in the 5-HT_{1A}R but opposite to 5-HT_{1B}R KO mice (Malleret et al., 1999; Ramboz et al., 1998; Zhuang et al., 1999), suggesting a complementary influence of these 5-HT receptors in regulating the different facets of anxiety.

In order to better understand the behavioural phenotype of 5-HT_4R KO mice and their response to chronic fluoxetine, we assessed the 5-HT_{1A}R functionality by performing *in*

611 *vivo and in vitro* techniques since this receptor subtype may critically intervene in the
612 efficacy of chronic antidepressant treatments, and in the neurobiology of depression
613 (Albert, 2012).

614 Similarly to the behavioural outcomes observed, chronic administration of fluoxetine
615 induced a desensitization of 5-HT_{1A}Rs in both 5-HT_4R KO and WT mice, an outcome
616 already reported in naïve animals treated with this antidepressant (Rainer et al., 2012).
617 This was evidenced by a reduced 8-OH-DPAT-induced hypothermia, though this effect
618 was less apparent in 5-HT_4R KO mice, suggesting a higher desensitization of 5-HT_{1A}Rs.
619 As discussed below, [^{35}S]GTPγS binding studies demonstrate increased basal binding
620 accompanied with a reduction in 8-OH-DPAT induced [^{35}S]GTPγS binding in the
621 dorsal raphe nucleus. These changes related to the functionality of presynaptic 5-
622 HT_{1A}Rs, though not discarding other adaptive mechanisms, may underlie this response
623 of 5-HT_4R KO mice in the 8-OH-DPAT-induced hypothermia test after the chronic
624 antidepressant treatment.

625 Also, 5-HT_4R KO mice showed a decreased 8-OH-DPAT-induced stimulation of
626 [^{35}S]GTPγS binding, consistently with a reduced concentration of 5-HT_{1A}Rs in the DRN
627 of these mutant mice (Conductier et al., 2006). An increased basal [^{35}S]GTPγS binding
628 was observed in 5-HT_4R KO mice, what might be due to a higher constitutive receptor
629 activity, including 5-HT_{1A}Rs. If this were the case, it could explain the hypersensitivity
630 of presynaptic 5-HT_{1A}Rs, and why citalopram is more efficient to inhibit the firing of 5-
631 HT neurons in 5-HT_4R KO mice than in their WT counterparts (Conductier et al.,
632 2006), though this hypothesis requests confirmation. In line with our results in 5-HT_4R
633 KO mice, reduced levels of both presynaptic (DRN) and postsynaptic 5-HT_{1A}R have
634 been reported in the hippocampus in mice (Conductier et al., 2006), in *postmortem* brain
635 samples from patients with depression (Boldrini et al., 2008; López-Figueroa et al.,
636 2004) and in PET studies (Drevets et al., 2000; Drevets et al., 2007; Hirvonen et al.,
637 2008; Meltzer et al., 2004). Animal studies also describe a decline in 5-HT_{1A}R
638 expression or functionality in different rodent models of depression/anxiety- following
639 maternal deprivation (Leventopoulos et al., 2009), social defeat (Kieran et al., 2010),
640 chronic unpredictable stress (Bambico et al., 2009) and chronic corticosterone treatment
641 (Rainer et al., 2012). Although it deserves further investigation, these changes on 5-
642 HT_{1A}R in the DRN may represent an adaptive response to counterbalance the absence

643 of the positive 5-HT$_4$Rs feedback on the firing activity of DRN serotonergic neurons
644 (Conductier et al., 2006; Lucas and Debonnel, 2002; Lucas et al., 2005).

645 In using the OBX animal model, we further circumvented how the 5-HT$_4$Rs are
646 potentially involved in some traits of depression- and anxiety-like behaviour, providing
647 a first series of results. As mentioned above and recall here, OBX mediates a
648 depressive-like phenotype as well as other behavioural and neurochemical alterations
649 that can be reversed by chronic antidepressant treatment (Freitas et al., 2013; Linge et
650 al., 2013 and 2016; Machado et al., 2012; Song and Leonard, 2005). An earlier study
651 shows an increase in the concentration of 5-HT$_4$Rs in the hippocampus in OBX mice
652 (Licht et al., 2010). However, our study shows that the constitutive absence of 5-HT$_4$Rs
653 did not modify the OBX-induced syndrome. In fact, 5-HT$_4$R KO mice presented a
654 similar behavioural outcome than WT counterparts following OBX (locomotor
655 hyperactivity and anxiety-like behaviour in the open-field, thus showing the same
656 susceptibility to the development and manifestations in this animal model of depression.

657 The major finding of our study is that chronic fluoxetine was not effective in attenuating
658 OBX-induced hyperactivity in 5-HT$_4$R KO mice, demonstrating its lack of
659 antidepressant effect since the reversal of OBX-induced hyperactivity is meant to have
660 high predictive validity (Freitas et al., 2013; Linge et al., 2013 and 2016; Machado et
661 al., 2012; Song and Leonard, 2005). Consistently with our results, a previous study in
662 non-transgenic mice (Mendez-David et al., 2014) showed that, following chronic
663 corticosterone treatment, some anxiolytic/antidepressant effects of fluoxetine are
664 prevented by chronic administration of a selective 5-HT$_4$Rs antagonist. However, as
665 stated above, chronic treatment with fluoxetine induced clear behavioural effects not
666 only in WT but also in 5-HT$_4$R KO mice under basal conditions.

667 At a molecular level, increased levels of BDNF and Arc mRNA associated with reduced
668 levels of trkB mRNA in non-OBX 5-HT$_4$R KO mice (basal condition) suggest adaptive
669 mechanisms that may likely limit major depressive- and anxiety-like behaviour in these
670 KO mice. Indeed, these molecular factors are well known to influence these behavioural
671 traits (see reviews by Castrén and Rantamäki, 2010; Li et al., 2015). Following OBX
672 surgery, 5-HT$_4$R KO mice treated with fluoxetine did not show the same regulation than
673 WT counterparts in BDNF and Arc expression in the hippocampus. The differences in
674 both the BDNF and Arc mRNA expression detected in the hippocampus of mice of both

675 genotypes could partly underlie the absence of efficacy of fluoxetine in modifying
676 locomotion in OBX-5-HT$_4$R KO mice (present study). Consistently, Freitas et al. (2013)
677 reported that the behavioural effects of chronic fluoxetine in OBX female Swiss mice,
678 are associated with molecular changes (regulation of ERK1/CREB/BDNF) in the
679 hippocampus. Our results suggest that the 5-HT$_4$Rs control of both the BDNF mRNA
680 expression in the DG, and CA3, and Arc mRNA expression in the CA1 can be
681 implicated in these molecular substrates, which can favor the antidepressant effect of
682 fluoxetine. Indeed, Imoto et al. (2015), using 5-HT$_4$R KO mice, introduced a potential
683 role of the 5-HT$_4$Rs in chronic fluoxetine treatment-induced neurogenic activity and
684 granule cell dematuration in the DG.

685 Both BDNF and its trkB receptor are implicated in mood disorders (Duman and
686 Monteggia, 2006). Decreased levels of BDNF and trkB mRNA are observed in the
687 hippocampus and frontal cortex in *postmortem* brain samples from patients with
688 depression (Dwivedi et al., 2003; Thompson et al., 2011), and a positive correlation
689 between BDNF serum levels and antidepressant responses was reported in individuals
690 with depression (Brunoni et al., 2008; Sen et al., 2008). Accordingly, chronic stress, a
691 risk factor of major depression, induced a decrease in the expression of BDNF in the
692 hippocampus in animals (Smith et al., 1995). A decreased expression of hippocampal
693 BDNF has been described in the OBX mouse model (Nakagawasai et al., 2016). In
694 contrast, chronic antidepressant treatments (fluoxetine, reboxetine) provoked increases
695 in the levels of BDNF in the hippocampus (Baj et al., 2012). Moreover, BDNF mimics
696 antidepressant-like effects in several behavioural experimental paradigms (Grønli et al.,
697 2006; Murakami et al., 2005). However, the implication of BDNF in anxiety- and
698 depressive-like behaviour is complex and can be contradictory. For instance, reduced
699 BDNF expression in the hippocampus is not associated with a depressive-like
700 phenotype (Taliaz et al., 2010), but with the OBX-depressive behaviour (Hendriksen et
701 al., 2012) in rats. Nonetheless, anhedonia and increased levels of BDNF observed in 5-
702 HT$_4$R KO mice are consistent with the increased hippocampal BDNF expression in
703 mice subjected to chronic unpredictable mild stress (Boulle et al., 2014) and OBX
704 (Hellweg et al., 2007).

705 This is the first time in which Arc signalling is studied in OBX animals chronically
706 treated with fluoxetine, and the literature on this topic is quite controversial (reviewed in
707 Li et al., 2015). For instance, low levels of Arc mRNA were reported in the frontal

cortex and the hippocampus following chronic social isolation stress in mice (Ieraci et al., 2016), but increased levels were found in rats following social defeat (Coppens et al., 2011) and in mice subjected to chronic unpredictable mild stress (Boulle et al., 2014). Pharmacological studies have reported that chronic SSRI treatment stimulates Arc mRNA expression in the cingulate and orbital frontal cortices in rats without producing any change in the hippocampus (De Foubert et al., 2004), and that chronic treatment with agomelatine normalized CUMS-induced increases in the levels of Arc mRNA in the hippocampus (Boulle et al., 2014). It can be speculated that the increased levels in Arc mRNA due to the absence of 5-HT_4Rs could represent a compensatory mechanism for the lifelong loss of 5-HT_4Rs. It has been reported that 5-HT_4R KO mice exhibit an increased muscarinic neurotransmission (Segu et al., 2010), which may account for the increased levels of Arc (and BDNF). Indeed, a direct relationship between cholinergic transmission and these neuroplasticity proteins has been reported regarding spatial memory acquisition (Gil-Bea et al., 2011).

In conclusion, our study shows that the absence of 5-HT_4Rs modulates the response of mice in depression- and anxiety-like experimental paradigms and did not influence the behavioural effects of chronic fluoxetine treatment. However, fluoxetine failed to reverse OBX-induced syndrome in 5-HT_4R KO mice, a response classically associated with differential effects in hippocampal neuroplasticity biomarkers. These results demonstrate that the absence of 5-HT_4Rs triggers adaptive changes that could maintain a global adaptive emotional state with the exception of anhedonia and a context-dependent anxiety. These findings further unmask that the behavioural and molecular effects of fluoxetine under pathological depression appear to be critically dependent on 5-HT_4Rs.

Disclosure

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

1. METHODS

1.1. 5-HT_4 receptor stimulated adenylate cyclase assay

5-HT_4 receptor stimulated adenylate cyclase assays were carried out as previously described by Vidal with slight modifications (Vidal et al., 2009). Striatal tissue samples were homogenised (1:120 w/v) in 20 mM Tris-HCl, 2 mM EGTA, 5 mM EDTA, 320 mM sucrose, 1 mM dithiothreitol (DTT), 25 µg/mL leupeptin, pH 7.4 and centrifuged at 500xg for 5 min at 4°C. The supernatants were centrifuged at 13000xg for 15 min at 4°C and the pellets were resuspended in 20 mM Tris-HCl, 1.2 mM EGTA, 0.25 M sucrose, 6 mM $MgCl_2$, 3 mM DTT and 25 µg/mL leupeptin. Membrane homogenates were pre-incubated for 5 min at 37ºC in reaction buffer (75 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.3 mM EGTA, 60 mM sucrose, 1 mM DTT, 0.5 mM 3-isobutylmethylxanthine, 5 mM phosphocreatine, 50 U/mL creatine phosphokinase and 5 U/mL myokinase) and 25 µl of either water (basal activity) or the 5-HT_4 agonists zacopride (10 µM). The reaction was started by the addition of 0.2 mM Mg-ATP and incubated at 37°C for 10 min. The reaction was stopped by boiling the samples for 4 min and then centrifuged at 13000xg for 5 min at 4°C. cAMP accumulation was quantified using a Cyclic AMP Competitive ELISA Kit (Thermo Fisher Scientific, MA, USA). Membrane protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Munich, Germany) using γ-globulin as standard.

1.2. Behavioural tests

The **open-field test** was performed as previously described (Linge et al., 2013 and 2016). The apparatus consisted in a wooden box (50 cm x 50 cm x 30 cm) with the centre of the arena highly illuminated (400 lux). Mice were placed in a corner of the open-field and allowed to freely explore it for 5 min. Mice behaviour was automatically video-tracked and analysed using the Any-maze software (Stoelting Co., USA). The total distance travelled, distance travelled in the periphery, time spent in the central zone, and distance travelled in the central zone were measured.

The **light-dark box test** was performed as previously described by Clément (Clément et al., 2009). The apparatus consisted of a shuttle box were the chambers (40 cm x 20 cm x 35 cm) were separated by a small door. One chamber was illuminated with a high

intensity light (400 lux) whereas the other was dark. Mice were individually placed on the dark side. The time and number of entries into each zone were recorded (Any-maze software).

The sucrose intake test was performed as previously described by Linge (Linge et al., 2013). Mice were deprived of any drink solution for 24 hours and subsequently each animal was given free access to a sucrose solution (1%) for 1 hour. The volume (ml) consumed by each animal were measured.

The forced swimming test (FST) was performed as previously (Porsolt et al., 1977). The mice were individually placed in a glass cylinder (height 24 cm, internal diameter 12 cm) filled with water at 25°C. The mice were left in the cylinder and the immobility time during the last four minutes of a 6 min session was measured (Any-maze software). Immobility time was considered when mice were floating and with minimal movements to keep the head outside the water. Climbing time was considered when mice produce active vigorous movements with the forepaws in and out of the water, and swimming time was considered when mice produce movement usually horizontal throughout the glass cylinder. Three behaviours were manually scored by a trained observer in blind conditions using the videotaped FST sessions.

The novelty suppressed feeding (NSF) was performed as previously described (Linge et al., 2013). Briefly, the mice were food-deprived 24 hours and only water was available. The day of the experiment, each mouse was placed into an open-field (50 cm x 50 cm x 30 cm; luminance 40-50 lux) containing a wood chip bedding with a food pellet (2 g) placed in the centre. The latency (in seconds) to eat the pellet was recorded (maximum 10 min) with the aid of Any-maze Video-tracking software Stoeling Co., USA. Immediately after an eating event, the mouse was placed into the home cage and allowed to feed freely for 5 minutes, and the amount of food consumption was measured (food consumption post-test).

The nesting test was adapted from Deacon (Deacon, 2006). In the test day, mice were individually housed and a 5 cm square of cotton were placed in every cage at the beginning of dark phase. After 12 hours, the nest score was evaluated by using the rate scale ranged between 1 and 5 where a score of 1 represents intact cotton or no nest produced, and 5 score is a perfect nest.

1.3. In vitro experiments

In situ hybridization. The protocol was adapted from Castro (Castro et al., 2003a). Cryostat sections were thaw-mounted onto slides and pre-treated for *in-situ* hybridization. Oligonucleotides complementary to BDNF mRNAs 5'-GGTCTCGTAGAAATATTGGTTCAGTTGGCCTTTTGATACCGGGAC-3' (Vaidya et al., 2001) and trkB mRNAs 5´-CCTTTCATGCCAAACTTGGAATGTCTCGCCAACTTG- 3' (Madhav et al., 2001) and Arc 5'-GCAGCTTCAGGAGAAGAGAGGATGGTGCTGGTGCTGG-3' (Kelly et al., 2008), were 3´end-labelled with [^{35}S]dATP using terminal deoxynucleotide transferase and added 250000 c.p.m./slide, with hybridization buffer (50% deionized formamide, 4x standard saline citrate (SSC), sodium phospate 10 mM pH 7.0, sodium pyrophosphate 1 mM, 10% dextran sulphate, 5x Denhardt's solution, 200 µg/ml salmon sperm DNA, 100 µg/ml poly A, heparin 0.12 mg/ml and 20 mM dithiothreitol). After incubation at 42°C for 16 hours, slides were washed at 50°C in 2x SSC buffer with DTT 1 M twice for 30 minutes followed by three washes of 5 minutes at room temperature with 1x SSC, 0.1x SSC, and ethanol 80% consecutively. Finally, slides were washed in ethanol 96% for 1 minute at room temperature. Sections were air-dried and exposed to film BioMax MR (Carestream) together with ^{14}C microscales at -20^{o}C for 3 weeks. The control of specificity was done with the probe without labelling (at a concentration 1000 times higher). The abundance of mRNA in selected areas was analysed and quantified using Scion Image Software. Optical density values were calibrated using ^{14}C microscales.

Autoradiography of protein G coupled to 5-HT$_{1A}$ receptors. Labelling of brain sections with [^{35}S]GTPγS was carried out as described previously (Castro et al., 2003b). Slide-mounted sections were pre-incubated for 30 min at room temperature in a buffer containing 50 mM Tris-HCl, 0.2 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM dl-dithiothreitol and 2 mM GDP at pH 7.7. Slides were subsequently incubated, for 2 h, in the same buffer containing adenosine deaminase (3 mU/ml) with [^{35}S]GTPγS (0.04 nM) and consecutive sections were co-incubated with 8-OH-DPAT (10 µM). The non-specific binding was determined in the presence of 10 µM guanosine-5-O-(3-thio) triphosphate (GTPγS). After the incubation, the sections were washed twice for 15 min in cold 50 mM Tris-HCl buffer (pH 7.4) at 4°C, rinsed in distilled cold water and then dried under a cold air stream. Sections were exposed to film BioMax MR (Carestream)

together with ^{14}C microscales at 4ºC for 2 days. Selected areas were analysed and quantified using Scion Image Software. Optical density values were calibrated using ^{14}C microscales.

BrdU Immunohytochemistry. BrdU staining was performed as previously described (Mostany et al., 2008). Free floating coronal sections were incubated for 2 h in 50% formamide/2x SSC at 65°C, followed by incubation in 2N HCl for 30 min. Then sections were incubated for 10 min in 0.1M borate buffer. After washing in PBS, sections were incubated in 1% H_2O_2 in PBS for 30 min to inactive endogenous peroxidase activity. After several rinses in PBS, sections were incubated in PBS/0.2% Triton X-100/5% goat serum (PBS-TS) for 30 min and then incubated with monoclonal mouse anti-BrdU (1:600; ref.: 11170376001 Roche Diagnostics, Barcelona, Spain) overnight at 4°C. After several rinses in PBS-TS, sections were incubated for 2 h with biotinylated goat anti-mouse Fab Fragment IgG secondary antibody (1:200; ref.: 115-066-006 Jackson ImmunoResearch Laboratories, Inc., US-PA), followed by amplification with avidin-biotin complex (Vector Laboratories). For quantification of $BrdU^+$ cells, one every sixth section throughout the hippocampus was processed and counted under a light microscope (Carl Zeiss Axioskop 2 Plus) at 40x and 100x magnification. The total number of $BrdU^+$ cells per section were determined and multiplied by 6 to obtain the total number of $BrdU^+$ cells per hippocampus.

2. RESULTS

2.1. Lack of 5-HT_4 receptor stimulated adenylate activity in 5-HT_4R KO mice

cAMP basal values in striatal membranes were similar in both genotypes (17.1 ± 1.7 *vs* 19.0 ± 1.9 pmol/min/mg protein for WT and 5-HT_4R KO mice, respectively). The 5-HT_4 agonist zacopride did not produce any change in 5-HT_4 receptor-induced cAMP accumulation in 5-HT_4R KO mice, (98.8 ± 15.1% zacopride-induced stimulation *vs* 100.0 ± 8.1% basal values) compared to the increase observed in WT mice (175.7 ± 26.7% zacopride-induced cAMP accumulation *vs* 100.0 ± 2.9% basal values; $*p < 0.05$), confirming the lack of 5-HT_4 receptors in these KO mice.

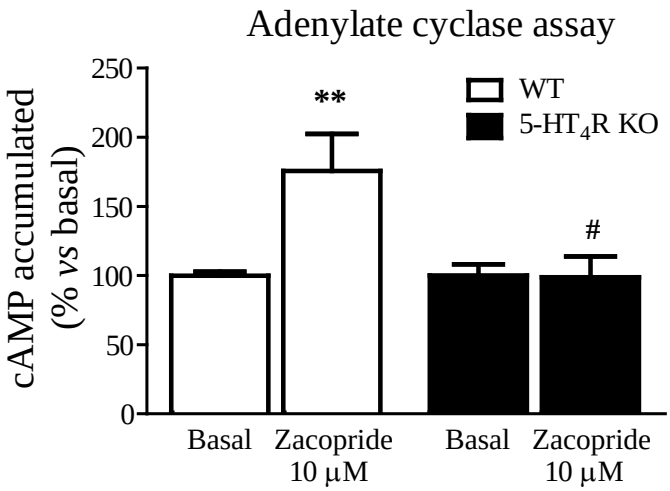

Figure S1. Absence of cAMP accumulation induced by zacopride (10 µM) in the striatum of 5-HT$_4$R KO mice. Two-way ANOVA analysis revealed main effect of genotype ($F_{(1,23)}$ = 6.8, $p < 0.05$), zacopride ($F_{(1,23)}$ = 6.4, $p < 0.05$) and genotype x zacopride interaction ($F_{(1,23)}$ = 7.1, $p < 0.05$). $^{**}p < 0.001$ *vs* WT- basal and $^{\#}p < 0.05$ WT-zacopride. Data are mean ± SEM, considering 100% the basal values, of duplicates from *n* = 5-7 mice per group.

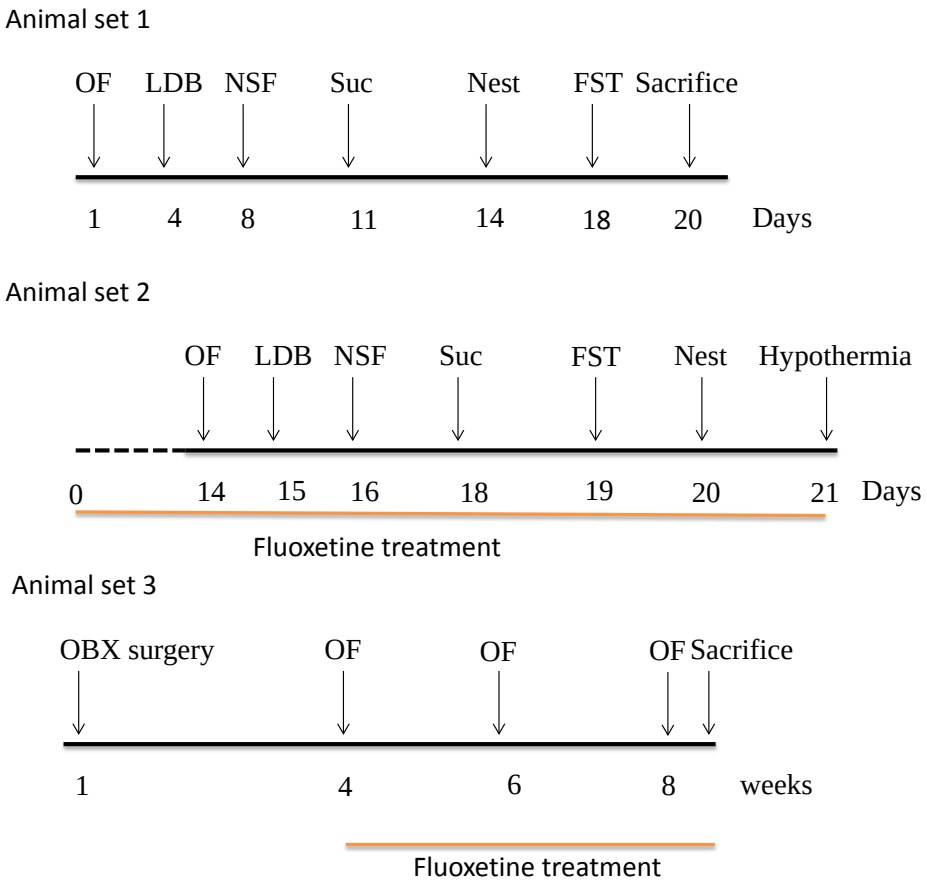

Figure S2. Behavioural testing schedule. OF: open-field; LDB: light-dark box; NSF: novelty-suppressed feeding; Suc: sucrose intake; Nest: nesting test; FST: forced swimming test; OBX surgery: olfactory bulbectomy surgery.

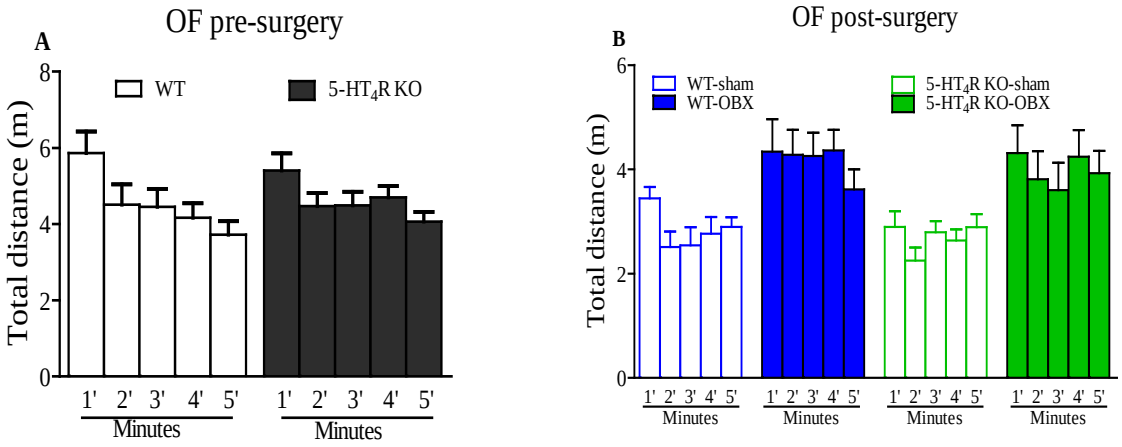

Figure S3. Temporal course of the total distance travelled in the open-field. Total distance travelled per one minute interval during a 5 min session, before **(A)** and at 4 weeks after OBX_surgery **(B),** in WT and 5-HT$_4$R KO mice. Data are mean ± SEM of n = 13-18 mice/group **(A),** and n = 7-8 mice/group **(B)**.

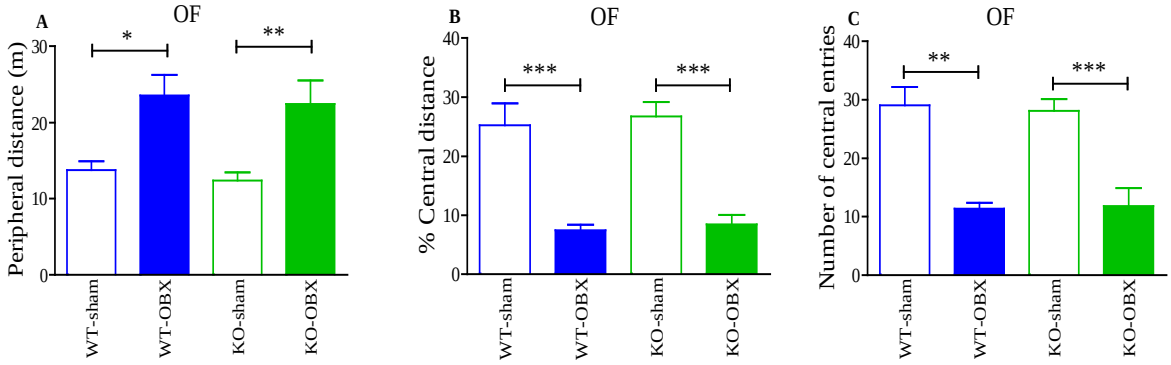

Figure S4. Effect of olfactory bulbectomy in peripheral and central activity in the open-field. Distance travelled at the periphery **(A)** % of distance travelled in the center **(B),** and number of entries in the central zone **(C)** in the open-field (5 min sesion) following 4 weeks of sham- and OBX surgery. Data are mean ± SEM of n = 7-8 mice per group. Two-way ANOVA revealed main effect of the surgery ($F_{(1,26)} = 18.0$, $p < 0.001$), ($F_{(1,26)} = 59.4$, $p < 0.001$), ($F_{(1,26)} = 42.0$, $p < 0.001$) on A,B and C respectively. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Newman-Keuls post hoc test).

Table S1. Statistical analysis report

Measurement	Statistical test	Comparison	Statistics	Degrees of freedom	*p*	Fig.
Central time OF	Two-way ANOVA	Treatment (F1)	F= 23.2	1, 58	< 0.001	**1A**
		Genotype (F2)	F= 5.9	1, 58	< 0.05	
		Interaction (F1xF2)	F= 0.1	1, 58	ns	
	Newman-Keuls multiple comparison test	WT *vs* KO			< 0.05	
		WT *vs* WT-flx			< 0.01	
		KO *vs* KO-flx			< 0.01	
Number central entries OF	Two-way ANOVA	Treatment (F1)	F= 34.2	1, 58	< 0.001	**1B**
		Genotype (F2)	F= 5.1	1, 58	<0.05	
		Interaction (F1xF2)	F= 0.00	1, 58	ns	
	Dunn's multiple comparison test	WT *vs* WT-flx			< 0.01	
		KO *vs* KO-flx			< 0.05	
Total distance travelled OF	Two-way ANOVA	Treatment (F1)	F= 9.0	1, 56	< 0.01	**1C**
		Genotype (F2)	F= 0.2	1, 56	ns	
		Interaction (F1xF2)	F= 0.00	1, 56	ns	
	Newman-Keuls multiple comparison test	WT *vs* KO			ns	
		WT *vs* WT-flx			ns	
		KO *vs* KO-flx			ns	
Time in light zone LDB	Two-way ANOVA	Treatment (F1)	F= 0.02	1, 50	ns	**1D**
		Genotype (F2)	F= 0.00	1, 50	ns	
		Interaction (F1xF2)	F= 0.2	1, 50	ns	
	Newman-Keuls multiple comparison test	WT *vs* KO			ns	
		WT *vs* WT-flx			ns	
		KO *vs* KO-flx			ns	
Latency to feeding NSF	Two-way ANOVA	Treatment (F1)	F= 14.4	1, 50	< 0.001	**1E**
		Genotype (F2)	F= 0.4	1, 50	ns	
		Interaction (F1xF2)	F= 0.00	1, 50	ns	
	Newman-Keuls multiple comparison test	WT *vs* KO			ns	
		WT *vs* WT-flx			< 0.05	
		KO *vs* KO-flx			< 0.05	
Post test NSF	Two-way ANOVA	Treatment (F1)	F= 0.2	1, 52	ns	**1F**
		Genotype (F2)	F= 0.2	1, 52	ns	
		Interaction (F1xF2)	F= 0.09	1, 52	ns	
	Newman-Keuls multiple comparison test	WT *vs* KO			ns	
		WT *vs* WT-flx			ns	
		KO *vs* KO-flx			ns	

Measurement	Statistical test	Comparison	Statistics	Degrees of freedom	*p*	Fig.
Sucrose intake	Two-way ANOVA	Treatment (F1)	F= 22.7	1, 60	< 0.001	**2A**
		Genotype (F2)	F= 3.5	1, 60	ns	
		Interaction (F1xF2)	F= 9.5	1, 60	< 0.01	
	Newman-Keuls multiple comparison test	WT *vs* KO			< 0.001	
		WT *vs* WT-flx			ns	
		KO *vs* KO-flx			< 0.01	
Nesting test	Two-way ANOVA	Treatment (F1)	F= 1.4	1, 63	ns	**2B**
		Genotype (F2)	F= 4.4	1, 63	< 0.05	
		Interaction (F1xF2)	F= 3.2	1, 63	ns	
	Dunn's multiple comparison test	WT *vs* KO			< 0.05	
		WT *vs* WT-flx			ns	
		KO *vs* KO-flx			ns	
Immobility time FST	Two-way ANOVA	Treatment (F1)	F= 21.6	1, 51	< 0.001	**2C**
		Genotype (F2)	F= 0.2	1, 51	ns	
		Interaction (F1xF2)	F= 0.09	1, 51	ns	
	Newman-Keuls multiple comparison test	WT *vs* KO			ns	
		WT *vs* WT-flx			< 0.01	
		KO *vs* KO-flx			< 0.01	
Swimming time FST	Two-way ANOVA	Treatment (F1)	F= 18.3	1, 51	< 0.001	**2D**
		Genotype (F2)	F= 0.7	1, 51	ns	
		Interaction (F1xF2)	F= 0.3	1, 51	ns	
	Newman-Keuls multiple comparison test	WT *vs* KO			ns	
		WT *vs* WT-flx			< 0.05	
		KO *vs* KO-flx			< 0.01	
Climbing time FST	Two-way ANOVA	Treatment (F1)	F= 20.7	1, 50	< 0.001	**2E**
		Genotype (F2)	F= 1.01	1, 50	ns	
		Interaction (F1xF2)	F= 0.2	1, 50	ns	
	Newman-Keuls multiple comparison test	WT *vs* KO			ns	
		WT *vs* WT-flx			< 0.001	
		KO *vs* KO-flx			< 0.05	

Measurement	Statistical test	Comparison	Statistics	Degrees of freedom	*p*	Fig.
8-OH-DPAT induced hypothermia	Two-way ANOVA	Treatment (F1)	F= 42.3	1, 19	< 0.001	**3**
		Genotype (F2)	F= 4.7	1, 19	< 0.05	
		Interaction (F1xF2)	F= 1.9	1, 19	ns	
	Newman-Keuls multiple comparison test	WT flx PAT *vs* KO-flx PAT			< 0.05	
		WT PAT *vs* WT-flx PAT			< 0.01	
		KO PAT *vs* KO-flx PAT			< 0.001	
Specific basal [^{35}S]GTPγS binding	Student *t*- test	WT *vs* KO DRN	t= 3.1	1, 12	< 0.01	**4 Table1**
		WT *vs* KO CxF	t= 2.9	1, 11	< 0.05	
Specific 8-OH-DPAT-induced [^{35}S]GTPγS binding	Student *t*- test	WT *vs* KO DRN	t= 2.7	1, 12	< 0.05	**4 Table1**
BDNF	Student *t*- test	WT *vs* KO DG	t= 2.5	1, 11	< 0.05	**5A**
TrkB	Student *t*- test	WT *vs* KO Amyg	t= 2.8	1, 11	< 0.05	**5B**
		WT *vs* KO CA1	t= 5.4	1, 12	< 0.01	
		WT *vs* KO CA3	t= 2.3	1, 12	< 0.01	
Arc	Student *t*- test	WT *vs* KO Cx-cing	t= 2.6	1, 12	< 0.05	**5C**
		WT *vs* KO CA1	t= 2.7	1, 12	< 0.05	
		WT *vs* KO CA3	t= 2.4	1, 12	< 0.05	
Total distance OF after surgery OBX	Two-way ANOVA	surgery (F1)	F= 9.4	1, 26	< 0.01	**6A**
		genotype (F2)	F= 0.3	1, 26	< 0.05	
		interaction (F1xF2)	F= 0.1	1, 26	ns	
	Newman-Keuls multiple comparison test	WT -sham *vs* WT-OBX			< 0.05	
		KO-sham *vs* KO-OBX			< 0.05	
Time in the center of OF after surgery OBX	Two-way ANOVA	Surgery (F1)	F= 35.04	1, 26	< 0.001	**6B**
		Genotype (F2)	F= 0.08	1, 26	ns	
		Interaction (F1xF2)	F= 0.9	1, 26	ns	
	Newman-Keuls multiple comparison test	WT -sham *vs* WT-OBX			< 0.001	
		KO-sham *vs* KO-OBX			< 0.001	

Measurement	Statistical test	Comparison	Statistics	Degrees of freedom	*p*	Fig.
Peripheral distance OF fluoxetine treatment	Two-way ANOVA	Time (F1)	F= 22.0	1, 26	< 0.001	
		Genotype (F2)	F= 1.5	1, 26	ns	
		Interaction (F1xF2)	F= 7.1	1, 26	< 0.01	
	Newman-Keuls multiple comparison test	WT-OBX *vs* WT-OBX flx 28d			< 0.05	**7A**
Peripheral distance per minute OF fluoxetine treatment	Two-way ANOVA	Time (F1)	F= 4.7	4, 52	< 0.01	
		Genotype (F2)	F= 6.6	1, 52	< 0.05	
		Interaction (F1xF2)	F= 1.2	4, 52	ns	
	Newman-Keuls multiple comparison test	KO-OBX flx 28d 1' *vs* KO-OBX flx 28d 5'			< 0.01	
		KO-OBX flx 28d 1' *vs* KO-OBX flx 28d 4'			< 0.01	
		KO-OBX flx 28d 1' *vs* KO-OBX flx 28d 3'			< 0.05	
		KO-OBX flx 28d 1' *vs* KO-OBX flx 28d 2'			< 0.05	**7B**

Measurement	Statistical test	Comparison	Statistics	Degrees of freedom	*p*	Fig.
BDNF expression in DG	Two-way ANOVA	Treatment (F1)	F= 16.1	1, 30	< 0.001	
		Genotype (F2)	F= 1.3	1, 30	ns	
		Interaction (F1xF2)	F= 1.3	1, 30	ns	
	Newman-Keuls multiple comparison test	WT-OBX *vs* WT-OBX flx 28d			< 0.05	
		KO-OBX *vs* KO-OBX flx 28d			ns	**8A**
BDNF expression in CA3	Two-way ANOVA	Treatment (F1)	F= 14.0	1, 31	< 0.001	
		Genotype (F2)	F= 5.7	1, 31	< 0.05	
		Interaction (F1xF2)	F= 0.7	1, 31	ns	**8B**

	Newman-Keuls multiple comparison test	WT-OBX *vs* WT-OBX flx 28d			< 0.05	
		KO-OBX *vs* KO-OBX flx 28d			ns	
BDNF expression in CA1	Two-way ANOVA	Treatment (F1)	F= 14.9	1, 32	< 0.001	**8C**
		Genotype (F2)	F= 0.6	1, 32	ns	
		Interaction (F1xF2)	F= 0.01	1, 32	ns	
	Newman-Keuls multiple comparison test	WT-OBX *vs* WT-OBX flx 28d			< 0.05	
		KO-OBX *vs* KO-OBX flx 28d			< 0.05	
Arc expression in DG	Two-way ANOVA	Treatment (F1)	F= 14.3	1, 30	< 0.001	**8D**
		Genotype (F2)	F= 2.4	1, 30	ns	
		Interaction (F1xF2)	F= 0.01	1, 30	ns	
	Newman-Keuls multiple comparison test	WT-OBX *vs* WT-OBX flx 28d			< 0.05	
		KO-OBX *vs* KO-OBX flx 28d			< 0.05	
Arc expression in CA3	Two-way ANOVA	Treatment (F1)	F= 16.5	1, 30	< 0.001	**8E**
		Genotype (F2)	F= 11.7	1, 30	<0.01	
		Interaction (F1xF2)	F= 0.5	1, 30	ns	
	Newman-Keuls multiple comparison test	WT-OBX *vs* WT-OBX flx 28d			< 0.05	
		KO-OBX *vs* KO-OBX flx 28d			< 0.05	
Arc expression in CA1	Two-way ANOVA	Treatment (F1)	F= 13.9	1, 31	< 0.001	**8F**
		Genotype (F2)	F= 5.9	1, 31	< 0.05	
		Interaction (F1xF2)	F= 1.7	1, 31	ns	
	Newman-Keuls multiple comparison test	WT-OBX *vs* WT-OBX flx 28d			< 0.01	
		WT-OBX flx 28d *vs* KO-OBX flx 28d			< 0.05	

Measurement	Statistical test	Comparison	Statistics	Degrees of freedom	*p*	Fig.
cAMP acumulation induced by Zacopride	Two-way ANOVA	Treatment (F1)	F= 6.4	1, 23	< 0.05	**S1**
		Genotype (F2)	F= 6.8	1, 23	< 0.05	
		Interaction (F1xF2)	F= 7.1	1, 23	< 0.05	
	Newman-Keuls multiple comparison test	WT-basal *vs* WT-zacopride			< 0.01	
		WT-zacopride *vs* KO-zacopride			< 0.05	
Total distance per minute OF	Two-way repeated measures ANOVA	Time (F1)	F= 9.7		< 0.001	**S3A**
		Genotype (F2)	F= 0.03		ns	
		Interaction (F1xF2)	F= 0.9		ns	
Total distance per minute after OB surgery in OF	Two-way repeated measures ANOVA	Time (F1)	F= 0.5		ns	**S3B**
		Genotype (F2)	F= 0.4		ns	
		Interaction (F1xF2)	F= 0.3		ns	
Peripheral distance in the OF after OBX surgery	Two-way ANOVA	Surgery (F1)	F= 18	1, 26	< 0.001	**S4A**
		Genotype (F2)	F= 0.3	1, 26	ns	
		Interaction (F1xF2)	F= 0.00	1, 26	ns	
	Newman-Keuls multiple comparison test	WT-sham *vs* WT-OBX			< 0.05	
		KO-sham *vs* KO-OBX			< 0.01	
% central distance in the OF after OBX surgery	Two-way ANOVA	Surgery (F1)	F= 59.4	1, 26	< 0.001	**S4B**
		Genotype (F2)	F= 0.3	1, 26	ns	
		Interaction (F1xF2)	F= 0.01	1, 26	ns	
	Newman-Keuls multiple comparison test	WT-sham *vs* WT-OBX			< 0.001	
		KO-sham *vs* KO-OBX			< 0.001	
Number of entries in the OF after OBX surgery	Two-way ANOVA	Surgery (F1)	F= 42.0	1, 26	< 0.001	**S4C**
		Genotype (F2)	F= 0.01	1, 26	ns	
		Interaction (F1xF2)	F= 0.06	1, 26	ns	
	Newman-Keuls multiple comparison test	WT-sham *vs* WT-OBX			< 0.05	
		KO-sham *vs* KO-OBX			< 0.001	